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Characterization of Ligand-induced Endocytosis
of EGF Receptors

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13. ABSTRACT (Maximum 200 words) Down-regulation of activated EGF-receptors (EGF-R) requires their tyrosine kinase (Y-kinase) activity. However, controversy existed as to whether ligand-induced activation of the EGF-R Y-kinase was required for internalization or for lysosomal targeting. We have addressed this issue using a cell-free assay which selectively measures the recruitment of EGF-R into coated pits. While EGF bound to wild-type receptors is efficiently sequestered in coated pits, sequestration of kinase-deficient receptors occurs inefficiently and at the same basal rate of endocytosis of unoccupied receptors or receptors lacking any cytoplasmic domain. Sequestration of deletion mutants of the EGF-R which lack autophosphorylation sites also requires an active Y-kinase. Our results directly establish that the EGF-R Y-kinase is required for internalization. They further suggest that a kinase substrate(s) other than the EGF-R itself, is required for its efficient ligand-induced recruitment into coated pits. Finally we have developed a powerful functional assay for identification of these substrate(s) based on our finding that the addition of a soluble EGF-R Y-kinase fully and specifically restores the recruitment of kinase-deficient EGF-R into coated pits.			
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FOREWORD

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Audra Schell 5/18/95
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(5) INTRODUCTION:

The binding of a peptide growth factor such as EGF to its cell surface receptor (the EGF-R) activates the receptor's internal tyrosine kinase (TK) domain and initiates a signal transduction pathway that ultimately leads to cellular proliferation (Carpenter, 1992). Ligand-binding to the growth factor-receptor also triggers its rapid entry into coated pits on the cell surface and subsequent internalization via clathrin-coated vesicles. Endocytosis of these growth factor-receptor complexes is a critical event for the attenuation of the mitogenic signal. Receptor defects or a defect in the endocytic apparatus may prevent this attenuation and contribute to uncontrolled cell proliferation and tumorigenesis. The molecular nature of the interactions between the activated growth factor receptors and the endocytic apparatus remains poorly understood. We have developed a novel and powerful cell-free system for the analysis of ligand-induced receptor-mediated endocytosis (Smythe and Schmid, 1991; Carter et al., 1993; Lamaze et al., 1993). The validity of this system for the specific and direct measurement of the early events of endocytosis has been well established both biochemically and morphologically (Schmid, 1993). Using this cell-free assay, we have demonstrated differential requirements for cytosolic factor(s) in the ligand-induced endocytosis of EGF-R relative to the constitutive endocytosis of nutrient receptors such as the transferrin-receptor complex (Tf-R) (Lamaze et al., 1993). We are currently using this system along with standard methods of protein purification and characterization to define the factor(s) required for the active recruitment of the EGF-receptor complex (EGF-R) to endocytic clathrin-coated pits.

Results from the past year's work provide an experimental basis for accomplishing the next year's objectives. We have found that kinase-defective EGF receptor (TK⁻-EGF-R, which contains a point mutation in the kinase domain) is not efficiently recruited to coated pits *in vitro* upon stimulation with EGF, but that recruitment can be restored *in trans* by the addition of purified soluble EGF receptor TK domain (Lamaze and Schmid, 1995). Together with our previous findings, these results suggest that a limiting cytosolic factor, regulated by tyrosine phosphorylation, may be essential for ligand induced recruitment of EGF-R into coated pits. The objectives of the next year's research are to identify the factor(s) required for ligand-induced recruitment of EGF-R into coated pit with the overall aim of elucidating the role of the EGF-receptor tyrosine kinase activity in endocytosis and receptor down-regulation.

(6) BODY (Experimental Methods)

The assay for ligand-induced recruitment of EGF-R into coated pits uses perforated mammalian cells expressing either wild type or mutant EGF receptors. The cells are depleted of their cytosol by low-speed centrifugation; the inner surface of the remaining plasma membrane as well as the cytoplasmic space are then accessible to exogenously added reagents including purified cytosol, antibodies, peptides. Perforated cells are resuspended in the presence of cytosol, an ATP-regenerating system and biotinylated EGF (B-EGF). Following incubation at 37°C the cells are returned to ice and incubated with avidin to mask any surface-accessible receptor-bound B-EGF. Excess avidin is quenched with biocytin and detergent lysates are prepared and plated onto microtitre wells coated with anti-EGF antibodies. Any remaining unmasked biotin residues on the captured EGF are quantitated with streptavidin-HRP and are taken as a measure of the recruitment to and sequestration of receptor-bound B-EGF into deeply invaginated and constricted coated pits (Lamaze et al., 1993). Active, cytosol-depleted membranes can be prepared, frozen and stored at -70°C providing a rapid, reproducible and quantitative assay.

The finding that the inefficient recruitment of TK⁻ EGF-R into coated pits *in vitro* can be fully restored *in trans* by the addition of soluble, constitutively active EGF-R tyrosine kinase (Lamaze and Schmid, 1995) provides a rapid and quantitative assay for identification of the relevant kinase substrate(s) involved. We have obtained recombinant baculovirus encoding the core TK domain of the EGF receptor tagged on its N-terminus with his₆ (designated, his₆-CTK). We are currently optimizing the large-scale production and purification of his₆-CTK and have found that it can be readily purified to near homogeneity and in large yield from infected Sf9 cells. The purified His₆-TK is active in phosphorylating known EGF-R kinase substrates and cytosolic proteins *in vitro* and can restore efficient sequestration of TK-EGF-R. Conditions for storage and maintenance of activity which are compatible in our assay system (e.g. glycerol inhibits our assay but stabilizes the his₆-CTK to freezing) are being established empirically. However, we expect that this reagent will be available within the next few months as a ready and reproducible source of the appropriate kinase activity to pursue the objective of identifying and isolating the EGF-R TK substrate(s) required for ligand-induced endocytosis of activated EGF-R.

Autophosphorylation of tyrosines located in the regulatory domain of the EGF-R are believed to result in a conformational change which exposes endocytic codes for recognition by the endocytic machinery and/or allow the recruitment of essential components of the endocytic machinery to the EGF-R (Sorkin and Waters, 1993). Therefore, while it is likely that one TK substrate required for EGF-R endocytosis is the EGF-R itself, other evidence suggests that it is not sufficient. For example, removal of the regulatory domain and all its autophosphorylation sites results in a truncated EGF-R (referred to as DR-TK^{+/or -}) whose internalization in intact cells (Chang et al., 1993) and in perforated cells (Lamaze and Schmid, 1995) is still dependent on an active tyrosine kinase domain. To identify whether membrane or cytosolic substrates or both are required to restore recruitment of TK⁻ EGF-R *in vitro*, each fraction will be independently treated with kinase. The EGF-R his₆-TK which will subsequently be removed by nickel chromatography (from the treated cytosolic fraction) or by pelleting the treated membrane fraction. These phosphorylated fractions will be re-incubated in an endocytosis assay with the treated or untreated reciprocal fraction to determine whether recruitment of TK⁻ receptors has been restored. These experiments will also be performed with cells expressing the DR-TK⁺ or DR-TK⁻ mutant EGF-R. In this case, it is likely that treatment of cytosol alone with EGF-R His₆-TK will be sufficient to restore the recruitment of DR-TK⁻ receptors. Based on our previous results and data in the literature, we anticipate that both the EGF-R and an addition cytosolic factors are substrates for the EGF-R kinase required for efficient recruitment of EGF-R into coated pits.

To identify the cytosolic factor(s) required for TK-dependent recruitment of TK⁻ receptors into coated pits, large quantities of cytosol will be activated with EGF-R his₆-TK and kinase substrates will be enriched by anti-phosphotyrosine affinity chromatography (commercially available). All chromatographic fractions will be tested for their ability to restore recruitment of DTK-EGF-R into coated pits. Untreated cytosol will be included in the assay to fulfill any requirements for other factors. Further controls for these experiments include measuring the basal rate of EGF-receptor endocytosis by the use of an anti-EGF receptor monoclonal antibody (mAb528) which does not activate the TK domain upon binding to the receptor, and by the use of a truncated EGF receptor that has only the first three amino acids of its cytoplasmic tail. To identify factors specifically required for ligand-induced recruitment of EGF receptors into coated pits, active cytosolic fractions will also be tested for their ability to support Tf-R recruitment. Standard

chromatographic methods will be subsequently employed to further purify and characterize the factor(s). All purification steps will be performed in the presence of phosphatase inhibitors. If the stability of the tyrosine phosphate is a problem alternate approaches would be to use ATPgS as a substrate for phosphorylation or to fractionate the cytosol *first* and *then* to treat individual fractions with the his6-TK before testing them in the EGF-R TK⁻ recruitment assay.

(7) CONCLUSIONS

By specifically and directly measuring the early events of EGF-R endocytosis, the experiments described above will provide important insight into the mechanisms that regulate EGF-dependent receptor endocytosis and its associated mitogenic signals. Since the rate of internalization is a key regulator to the attenuation of mitogenic signalling by the EGF-R, identification of the kinase substrate necessary for rapid and efficient recruitment of activated EGF-R into coated pits will provide an important target for therapeutic intervention or potentially for diagnostic purposes. In the long-term, understanding how endocytosis might regulate cell proliferative responses will be important not only for therapeutically manipulating the unregulated response of breast (and other) cancers to growth factors but may have far reaching implications in other proliferative diseases.

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Addendum to Annual Report for Grant No.: DAMD17-94-J-4031

The following indicates the progress made during last funding period (July 1994-June 1995) relative to our initial Statement of Work.

Task 1: Establishing and optimizing the assay for the EGF-R tyrosine kinase dependent recruitment of EGF-R into coated pits.

Our initial estimates for completion of this task within 6 months were over-ambitious although the following indicates that this task has been successfully completed within the first year of funding.

a) Optimization of His₆-tagged EGF-R kinase expression in baculovirus infected cells; and, b) Purification of the His₆-tagged core kinase

Initial attempts to express and purify this construct using recombinant baculovirus obtained from Gordon Gill's lab proved unsuccessful, despite the fact that considerable effort and time (4 months) was applied towards this goal. In fact, the Gill lab has also been unable to purify large quantities of active kinase using this construct. Problems encountered included: 1) low levels of expression (despite our efforts to re-plaque purify the virus and to obtain new high-titre virus stocks), 2) low yields and ineffective purification by Nickel-chelate chromatography and 3) the purified kinase was inactive and aggregated. Neither we nor Gordon Gill's lab were able to overcome these problems. At this point we became aware of an alternate His₆-core kinase baculovirus recombinant

generated by Dr. J. G Koland (U. of Iowa). He generously provided us with this recombinant baculovirus and after generating our own high titre stock we were now able to obtain reasonable levels of expression and to purify active His₆-core kinase by Nickel Chelate chromatography. A representative purification is shown in Figure 1. We have also had to empirically optimized conditions for storage so that accomplishing the goal of a reproducible, active and readily available source of core kinase has taken most of the first year. Kinase preparations now have high specific activity and can be stored at -70°C.

In our initial proposal we showed that a soluble constitutively active EGF-R kinase encoded by the entire cytoplasmic domain of the EGF-R could restore efficient recruitment of kinase-deficient (TK⁻) EGF-R into coated pits when added *in trans* to our cell-free assay. In subsequent experiments completed before the grant was activated and recently published (Lamaze and Schmid, 1995, *J. Cell Biol.* 129:47-54) we found, however that this restoration showed a sharp optimum and stimulation was abrogated

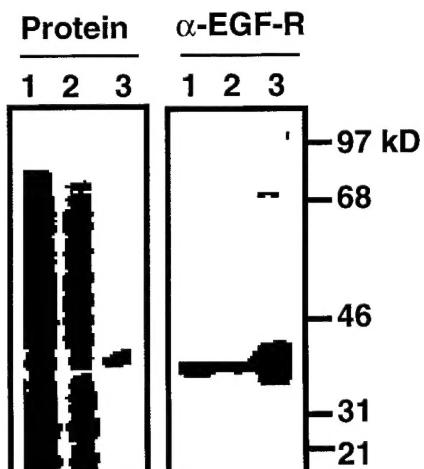


Figure 1. Expression and purification of His₆-tagged EGF-R core kinase domain in Sf9 cells.
Recombinant baculovirus encoding the core EGF-R kinase domain (aa645-971) His₆-tagged at its N-terminus were used to infect Sf9 insect cells for expression of core EGR-R kinase domain. Lane 1: Cell lysate, Lane 2: Ni-chelate flow through, Lane 3: purified core kinase. A polypeptide of ~38 kD which immunoreacts with polyclonal anti-EGF-R antibody was purified from cytosolic fraction.

at higher concentrations of intact EGF-R cytoplasmic domain (see Figure 2).

A likely explanation for this inhibitory effect was that the endocytic codes found in the C-terminal regulatory domain of the EGF-R compete for limiting components of the

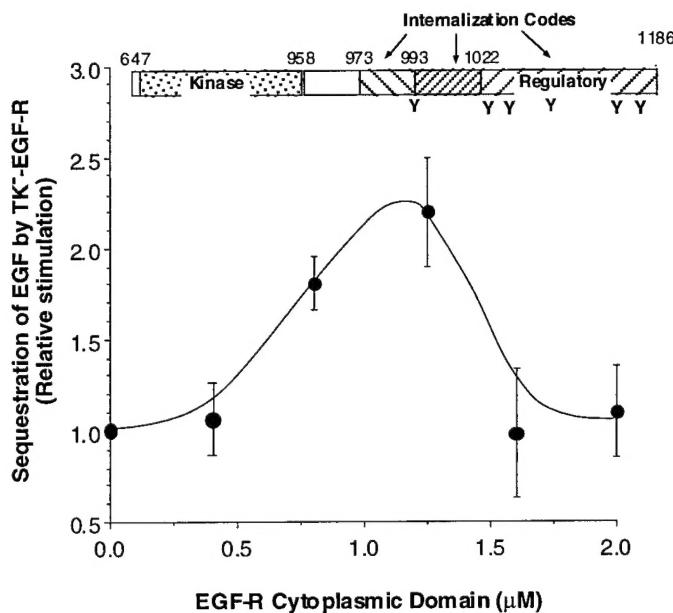


Figure 2: Restoration of sequestration of TK-EGF-R with the constitutively active kinase encoded by the soluble EGF-R cytoplasmic domain Note that at higher concentrations restoration is abrogated presumably due to inhibition by soluble internalization codes in the regulatory domain of the EGF-R.

endocytic machinery which recognize these codes on the intact membrane-associated EGF-R. We have confirmed this using the His₆-core kinase. Restoration by this construct is titratable, reaches saturation and shows no inhibition at higher concentrations (Figure 3a). This titration also establishes the activity of the recombinant His₆-core kinase preparation from baculovirus-

infected Sf9 cells. As further proof for the notion that soluble internalization motifs inhibit EGF-R recruitment into coated pits, we have obtained an additional construct

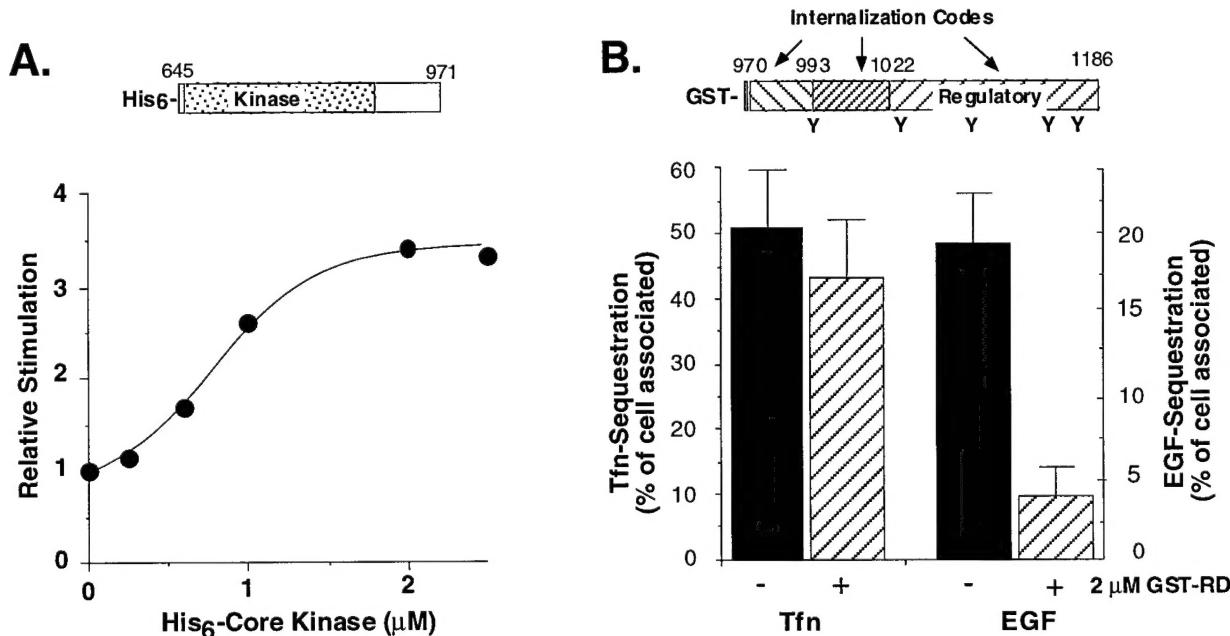


Figure 3. Dissection of regions of the EGF-R cytoplasmic domain that stimulate and inhibit EGF-R sequestration. **Panel A.** A constitutively active core EGF-R kinase domain restores efficient recruitment of TK-EGF-R when added *in trans* without the inhibitory effects associated with the intact cytoplasmic domain. **Panel B.** The EGF-R regulatory domain containing internalization sequences inhibits sequestration of wt-EGF-R without effecting Tfn-R uptake.

from Gordon Gill (UCSD) which encodes the C-terminal regulatory domain of the EGF-R fused to GST. We have expressed and purified this protein from *E. coli* and found, as predicted by our model, that it inhibits sequestration of wt EGF-R without affecting uptake of Tf_n receptors (Figure 3b). These results provide two independent means to identify the factor(s) required for EGF-R sequestration. In the coming year, attempts will be made to enrich the relevant tyrosine-kinase substrate by anti-phosphotyrosine affinity chromatography and/or by affinity chromatography on glutathione-Sepharose columns using the GST-regulatory domain constructs.

c. Optimizing the assay for the EGF-R tyrosine kinase-dependent recruitment of EGF-R into coated pits using the core kinase-domain and cells expressing kinase-deficient EGF-R lacking the inhibitory regulatory domain: c'958/f993-1022.

Attempts to use the EGF-R construct c'958/f993-1022 (i.e. a deletion at aa958 fused with the internalization sequence encoded by aa993-1022, as indicated in Fig 3) were unsuccessful for as yet unexplained reasons. However the construct $\Delta 991$ (a simple deletion of aa992-1186) which encodes the first internalization motif but lacks any

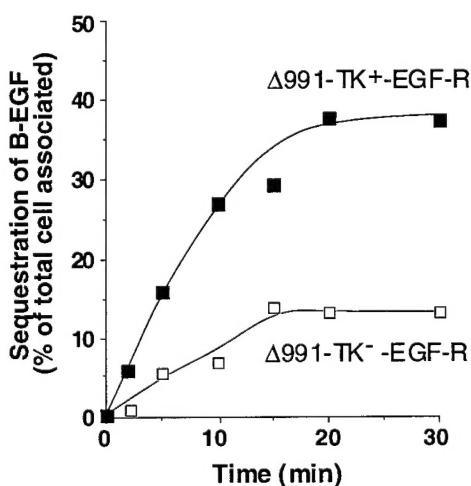


Figure 4 Time course of sequestration of B-EGF in perforated B82L cells expressing either $\Delta 991$ -TK⁺-EGF-R (■) or $\Delta 991$ -TK⁻-EGF-R (□). Sequestration is dependent on an active tyrosine kinase even though these truncated receptors lack any autophosphorylation sites.

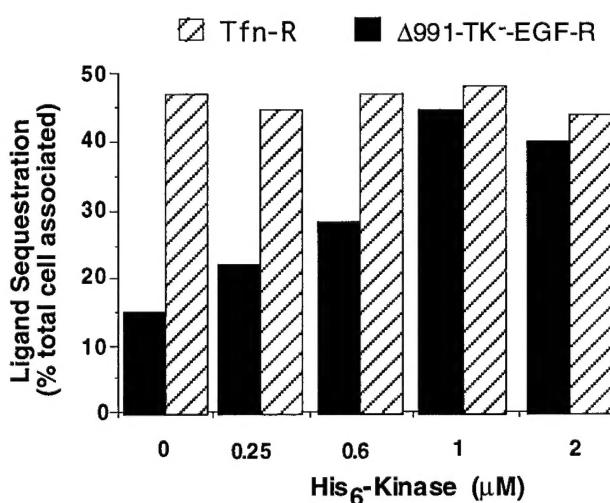


Figure 5: Core EGF-R kinase restores efficient sequestration of EGF in perforated cells expressing the $\Delta 991$ -TK⁻-EGF-R.

autophosphorylation sites did show kinase-dependent sequestration of EGF into coated pits *in vitro*. This result, again obtained after submission of the grant but before activation of funding has been published (Lamaze and Schmid, 1995). It is reproduced here (Figure 4) for your information as it represents completion of one of the objectives necessary to optimize our assay. The data shown in Figure 5 completes our optimization of this assay (Task 1) as it establishes that the core EGF-R kinase can restore efficient sequestration of EGF mediated by $\Delta 991$ -TK⁻-EGF-R.

In the next funding period of the grant we plan to pursue Tasks 2 and 3 as indicated in our initial Statement of Work.